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14. ABSTRACT
The cell of origin in clear cell renal cell carcinoma (ccRCC) is ambiguous. Previously, we had found through microarray "data mining", that Grainyhead-like-2 (GRHL2) was expressed at much lower levels in ccRCC compared with normal kidney. In light of the relatively high overall expression of GRHL2 in the kidney, compared with other organs, we interpreted this result to mean that GRHL2 was down-regulated during oncogenic transformation. Our recent data, however, indicate that the much more likely interpretation is that ccRCC arises from GRHL2-negative cells of origin. These data include: a. low or undetectable expression levels of GRHL2 in either normal proximal or distal tubule-derived cell lines; b. low immunohistochemical signal for GRHL2 in normal proximal or distal tubules of human kidney samples; c. very high signal for GRHL2 in collecting duct and ureter. These results are highly significant because they show that ccRCC arises uniquely from GRHL2-negative but not GRHL2-positive ductal epithelial cells, informing a novel understanding of the biology of ccRCC.

15. SUBJECT TERMS
Grainyhead-like-2/kidney/collecting ducts/proximal tubules/distal tubules/clear cell renal carcinoma

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1. INTRODUCTION: The original intent of our project was to test the role of an epithelial master-programming transcription factor called Grainyhead-like-2 (GRHL2) in preventing the Epithelial-to-Mesenchymal Transition that was believed to be a characteristic of clear cell renal carcinoma (ccRCC). We were also planning to test the role of GRHL2 in regulating anoikis in normal and transformed renal cells. New and surprising results were obtained, as described below, that inform a new perspective on this problem.

2. KEYWORDS: Anoikis, Epithelial-Mesenchymal Transition, clear cell renal carcinoma, Grainyhead-like-2, Von Hippel-Lindau protein, proximal tubules, distal tubules, collecting ducts/kidney

3. OVERALL PROJECT SUMMARY:

Objective 1. To determine whether GRHL2 is a tumor suppressor for RCC.

In breast cancer, we had published previously that GRHL2 is a suppressor of the oncogenic epithelial-mesenchymal transition (EMT) that is down-regulated specifically in a subclass of breast cancer ("claudin-low") that is characterized by widespread EMT (*1, 2*).

With regard to clear cell renal carcinoma (ccRCC), analysis of published microarray data using the GEO database indicated a significantly decreased expression of GRHL2 in ccRCC compared to normal kidney (figure 1). Moreover, abundant literature has characterized ccRCC as an EMT-derived tumor type (e.g., (*3, 4*)). Combining these two concepts, we hypothesized that: a. the VHL defect in most ccRCC directly or indirectly down-regulates GHRL2 expression; b. the down-regulation of GRHL2, as in breast cancer, is a critical step for tumor cell commitment to EMT and anoikis-resistance.

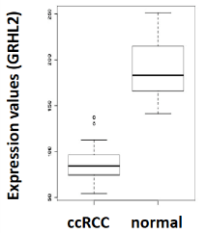


Figure1. Decreased expression of GRHL2 in ccRCC compared to normal kidney (*1*)

With this in mind, we obtained a collection of normal and transformed kidney cell lines and examined the expression of GRHL2 as well as the effect of ectopically expressing GRHL2 on normal vs. tumor phenotypes. This panel included: RCC4 (ccRCC) cells and RCC4 plus VHL re-expression (*5*); 786-0 ccRCC, 786 plus VHL re-expression (*6*); HKC-8 (normal proximal tubule cells), HK-2 (normal proximal tubule cells), UMRC3 (ccRCC), UMRC3 with re-expressed Soluble Frizzled Receptor-3 (*7*), mIMCD3 (inner medullary collecting duct epithelial cells, mouse) and MDCK (distal tubule or collecting duct, canine). The results of these studies were as follows:

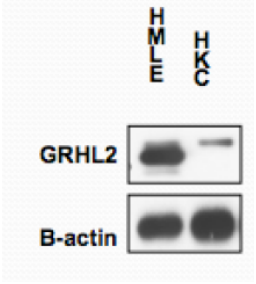


Figure 2a. Gel mobility of anti-GRHL2 reactive band differs in HMLE vs. HKC cells.

A. Initial preliminary data indicated that all these lines expressed GRHL2 to various extents on western blots, using a well-characterized "Atlas Project" GRHL2 antibody.

B. There were two problems with the identification of this western blot band as GRHL2, however. First, the band in HKC lysates migrated slightly slower than the known GRHL2 band in control (HMLE cell) lysates (figure 2a). Secondly, three different shRNAs directed against GRHL2 produced little or no knockdown of the band in HKC cells (figure 2b), while significantly knocking down the control GRHL2 band in HMLE cells (*1, 2*).

C. qRT-PCR data indicated lack of significant expression of GRHL2 mRNA in the cell lines listed above, except in the case of MDCK cells and mIMCD-3 cells – notably, both collecting duct-derived cell lines (although MDCK is sometimes cited as a distal tubule cell line).

D. By contrast, in the two collecting duct-derived cell lines, mIMCD3 and MDCK, GRHL2 was expressed at high levels, co-migrated with HMLE-derived GRL2 on a western blot and could be knocked down efficiently with GRHL2 shRNA (figure 4a, 4b and data not shown).

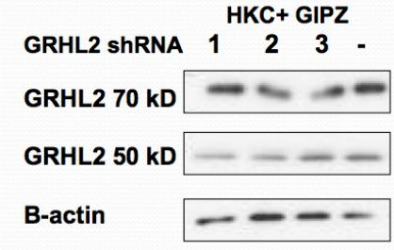


Figure 2b. GRHL2 shRNA only modestly reduces the 70 kDa band in HKC cells.

E. Examination of the Atlas database for tissue specific expression using validated antibodies indicated only weak expression in tubules, that was mostly cytoplasmic (note that GRHL2 is a nuclear transcription factor). By contrast, the urinary bladder (UB) which is of the same tissue origin and cell type (urothelial cells) as the collecting ducts, was highly positive for nuclear GRHL2 (figure 5).

In summary, these results indicate that renal cells derived from the metanephric mesenchyme – which include essentially all the cells within mature nephrons—are essentially negative for GRHL2 expression. These are the cells, however, that give rise to ccRCC. By contrast, the cells of the uretary tree/collecting duct (epithelial cells induced to form these structures by induction of GDNF/Ret signaling of the Wolffian duct during development) are GRHL2 positive and do not give rise to ccRCC. This raises the important question of whether GRHL2 expression is a potent protective factor against RCC, analogous to its tumor suppressor role in breast cancer.

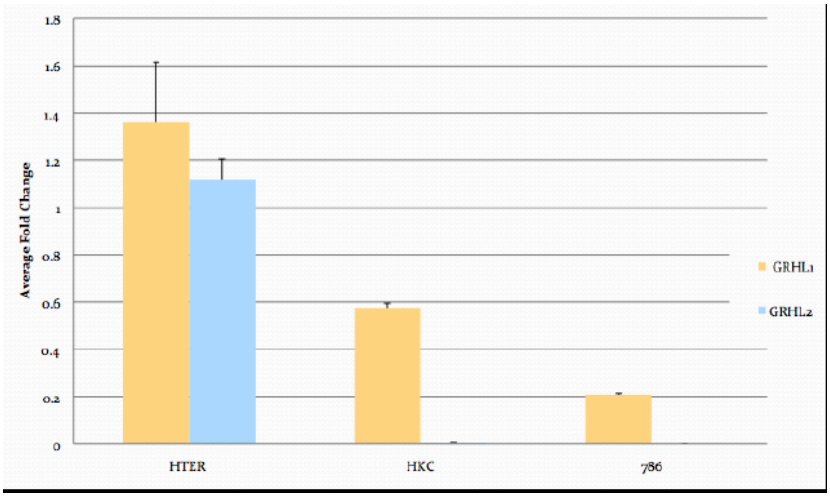


Figure 3. Lack of GRHL2 mRNA expression in HKC and 786 cells (qRT-PCR). Yellow bars: GRHL1, blue bars: GRHL2

Figure 4a. MDCK cell GRHL2 is knocked down by GRHL2 shRNA

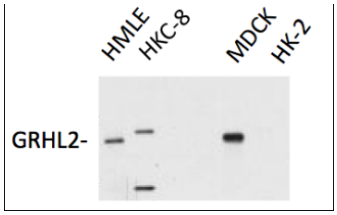


Figure 4b. MDCK GRHL2 co-migrates with HMLE GRHL2.

Objective 2. To determine how GRHL2 suppresses EMT, thus preventing RCC.

As explained above, the precursor cells for ccRCC are GRHL2-negative. Plausibly, it is this property that makes them susceptible to the partial EMT accompanying oncogenic transformation. We therefore examined the effects of GRHL2 expression on the responses of renal epithelial cells to TGF-b and HGF. The rationale for this was that both of these factors contribute significantly to ccRCC development, although TGF-b is a potent pro-fibrotic agent in the kidney while HGF suppresses fibrosis (*8-10*).

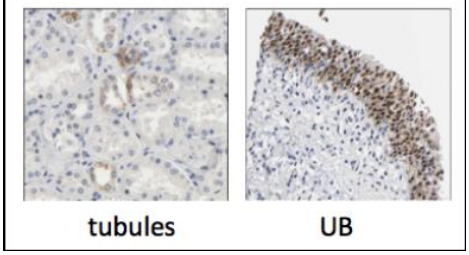


Figure 5. Expression of GRHL2 by IHC in kidney tubules and urinary bladder (data from http://www.proteinatlas.org/ENSG00000083307/normal)

Our results are summarized as follows:

A. Constitutive GRHL2 expression (by infection with a GRHL2 retroviral vector) suppressed the HGF- induced collagen gel invasion of 786-0 cells (figure 6).

B. In MDCK cells, HGF or constitutive GRHL2 expression each suppress the fibrosis/EMT markers, fibronectin and alpha-smooth muscle actin (figure 7). This indicates that pathways downstream of GRHL2 partially overlap with those downstream of HGF, both producing the effect of suppressing fibrotic/mesenchymal gene expression.

Objective 3. To determine how GRHL2 suppresses EMT-mediated anoikis-resistance, thus preventing RCC progression.

The initial phase of this aim was to test the effect of GRHL2 expression on anoikis sensitivity in normal or ccRCC cell lines. To initiate this study, we expressed GRHL2 in the (GRHL2-negative, VHL-negative) ccRCC cell line, 786-0 and assayed this pair of isogenic cell lines, together with the 786-0+VHL cell line. Interestingly, either re-expression of VHL or ectopic GRHL2 expression sensitized 786-0 cells to anoikis (figure 8).

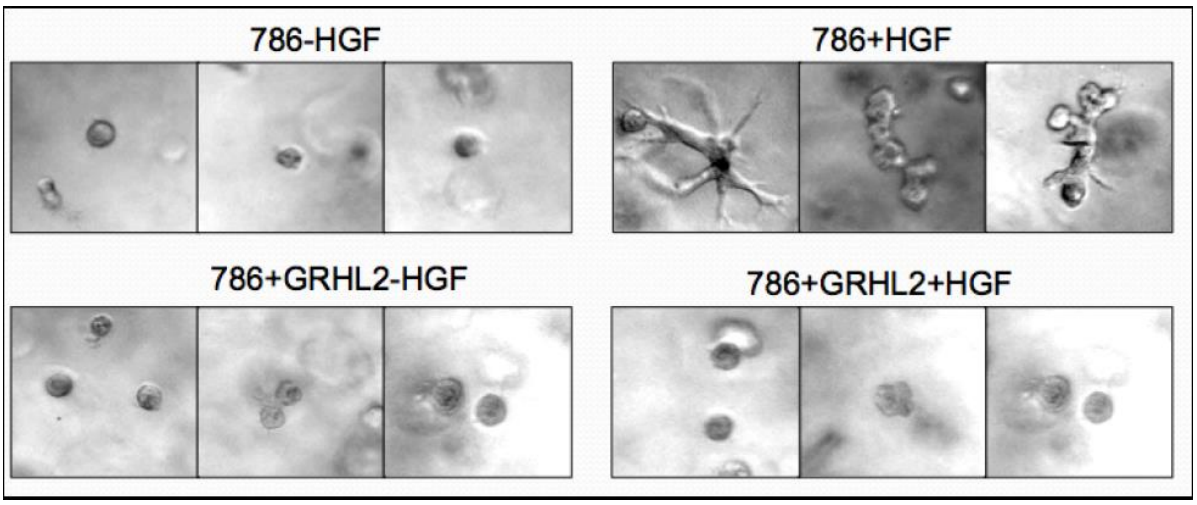


Figure 6. Constitutive GRHL2 expression suppressed the HGF- induced collagen gel invasion of 786-0 cells

Additionally, the constitutive expression of GRHL1, which was expressed endogenously in 786-0 cells at low levels (data not show), also sensitized the cells to anoikis (figure 9).

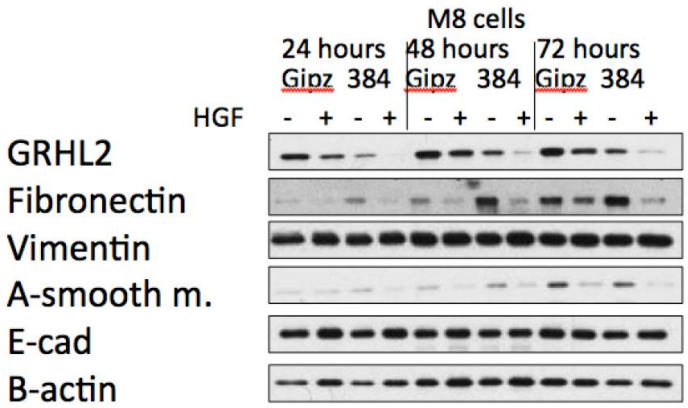
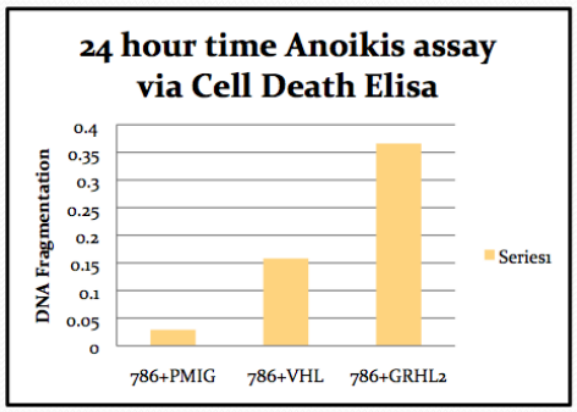


Figure 7. MDCK cells expressing control shRNA empty vector (pGIPZ) or GRHL2 shRNA (384) were treated with HGF for the indicated times, followed by western blotting for the EMT/fibrosis markers fibronectin, alpha-smooth muscle actin or vimentin.



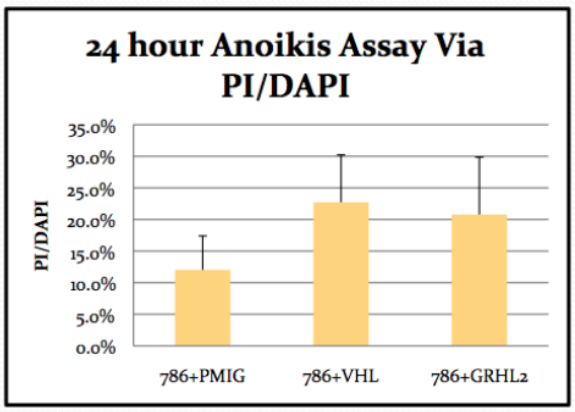


Figure 8. GRHL2 and VHL sensitize 786-0 RCC cells to anoikis. Assayed by Cell Death ELISA (left) or % PI positive (apoptotic) cells (right).

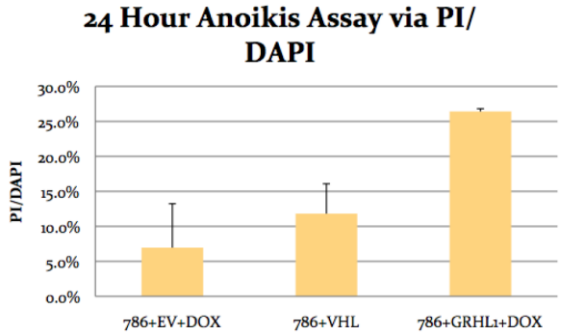


Figure 9. Constitutive expression of GRHL1 sensitizes 786-0 cells to anoikis.

4. KEY RESEARCH ACCOMPLISHMENTS:

- --Discovery that ccRCC cell lines arise from GRHL2-negative cells of origin within the kidney. This informs the novel question of how GRHL2 expression prevents tumor initiation or progression in ccRCC.
- --Discovery that GRHL2 suppresses anoikis in renal carcinoma
- --Discovery that GRHL2 suppresses collagen gel invasion in ccRCC cells
- --Discovery that GRHL2 suppresses fibrotic/mesenchymal gene expression in renal cells

5. CONCLUSION:

Clear cell RCC is thought to arise from proximal and/or distal tubules within the numerous nephrons of the kidney. Our major significant finding of the past year is that these cell types are, surprisingly, negative for GRHL2 expression, despite having other characteristics of epithelial cells. Interestingly, the anatomically close neighbor, collecting ducts, are highly GRHL2 positive and are not thought to give rise to ccRCC (in fact, they only rarely produce tumors). These results suggest that GRHL2 expression is highly protective against ccRCC. This positions GRHL2 as a tumor suppressor in renal carcinoma, analogous to the effect reported earlier in breast cancer. This will be pursued further by the experiments outlined in the grant application.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project.

Nothing to report

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report

8. REPORTABLE OUTCOMES:

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9. OTHER ACHIEVEMENTS:

Nothing to report

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11. APPENDICES: N/A